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14. ABSTRACT

The goal of this grant was to construct and test vectors that utilized the tetracycline repressor system and expression of short hairpin RNAs to regulate expression of genes in a conditional manner (plus or minus tetracycline) and to test the system in prostate cancer cell models to regulate genes of interest in prostate cancer. A series of vectors were constructed and tested for the regulation of Prostate Derived Factor (PDF) and the tumor suppressor p53. We did not see tetracycline-dependent inhibition of p53 expression. However, we found some LNCaP cell clones in which we saw up to 75% suppression of PDF expression. Further optimization of the vector did not generate clones with tighter regulation of expression. Thus, a retroviral vector directing tetracycline-regulated expression of a short hairpin RNA targeting specific genes was developed but proved only partially successful in achieving robust suppression of target genes in a tetracycline-dependent fashion.

15. SUBJECT TERMS
RNAi, vector, gene expression, tetracycline repressor, KRAB, HOX, PDF

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This was a Hypothesis Exploration award to develop and test vectors designed assist studies on the function of particular genes in prostate cancer cell models. To study the role of a particular gene, it can be critical to be able to perturb expression of that gene to study the effects of the gain or loss of expression. It is particularly useful to have a system wherein expression of specific genes can be regulated in a conditional manner. The goal of this grant was to construct and test vectors that utilized the tetracycline repressor system and expression of short hairpin RNAs to suppress expression of genes in a conditional manner (plus or minus tetracycline) and to test the system in prostate cancer cell models for regulation of genes of interest in prostate cancer.

Main Body: W81XWH-04-1-0205 Development of vectors for the regulated expression of RNAi

Construction of retroviral RNAi expression vector with mutated U6 promoters with embedded tetracycline operator (tetO) sequences was completed successfully. (task one)

Construction of retroviral RNAi expression vector expressing a chimeric tetracycline repressor-Kruppel Associated Box (KRAB) supersuppressor was completed successfully (task two). Figure 1 presents a schematic diagram of the complete vector pU6O/RK. This vector is the end product of the steps of tasks one and two and is the base vector into which targeting sequences are introduced. This vector is actually a series of three. The difference between the three is the dominant selectable marker offered in each making the vectors much more widely usable. The markers available are resistance to the antibiotics puromycin, hygromycin, and the neomycin derivative, G418.

Introduction of hairpin sequences for genes targeted for regulated suppression into the vector and confirmation of the vector sequence was addressed next. This was the goal of task three. This has bee completed for Prostate Derived Factor (PDF). In fact, two different vectors were constructed using different hairpin sequences to target PDF. In addition, RNAi vectors were made targeting the key tumor suppressor, p53, and, in collaborative studies, the coactivator protein BRG-1.

The fourth task was to assess the delivery and efficacy of regulatable suppression of PDF and p53. Retroviral vectors targeting PDF or p53 were packaged, harvested, and used to transduce prostate cancer cell lines. Individual clones surviving antibiotic selection indicating successful transduction with the RNAi vector were picked and expanded. Individual clones were treated with or without tetracycline and expression of the targeted gene assessed. A total of 7 LNCaP clones with targeted PDF were assessed. Figure 2 depicts the suppression of PDF expression in the first clone analyzed. Unfortunately, no clone was identified subsequently that showed repression of PDF markedly better than the initial clone. We also selected two clones derived from MDA-PCa2b cells transduced with pU6O/RK-PDF but neither of these showed good tetracycline-dependent

suppression of PDF expression. MDA-PCa2b cells, while robust expressers of PDF, grow very slowly making clonal isolation difficult.

Tasks accomplished beyond those set out in the original proposal:

Because the most regulated clones exhibited at best about 75% suppression, see Figure 2, we speculated that better suppression of gene expression might be achieved with a better targeting sequence. We, therefore, made a vector with a targeting sequence that had been widely used to suppress p53, a key tumor suppressor and regulator of PDF expression. Eleven clones were selected and screened but no clones exhibiting highly regulated expression of p53 were identified. We also tried a second PDF targeting sequence without improving the degree of PDF suppression we observed. More detail on these experiments follow. To address whether we could achieve higher levels of expression by selecting for clones that expressed high levels of the tetracycline repressor, we modified the regulatable RNAi retroviral vector by replacing the KRAB domain of the chimeric repressor with sequences encoding green fluorescent protein (GFP). This vector, pU6O/RG is exactly like the pU6O/RK shown in figure one except it encodes a tet-repressor/GFP fusion gene instead of a tet-repressor/KRAB fusion. Transduction of cells with this vector results in many cells exhibiting strong green fluorescence indicating robust expression of the tetracycline repressor-GFP fusion protein. Nonetheless, analysis of 12 clones with targeted PDF did not show suppression markedly better than those clones assessed initially. A different hairpin sequenced was designed and cloned into pU6O/RG. Cells were transduced and a dozen individual clones analyzed again without seeing data any better than shown in Figure 2. All together, about one-third of the clones examined exhibited significant suppression of PDF expression, another third showed weak suppression, and one third no significant suppression. At this time, we read a report suggesting that the widely used tet-operator sequences were not optimal and tighter regulation could be achieved by modifying the tet-operator sequences. We constructed an RNAi vector with the appropriate alterations to the tet operator site. At this point we have not introduced targeting sequences in order to test the efficacy of this last iteration of the RNAi vector.

Key Accomplishments: W81XWH-04-1-0205 Development of vectors for the regulated expression of RNAi

1. Construction of retroviral RNAi expression vector with mutated U6 promoters with embedded tetracycline operator (tetO) sequences. (completion of task one)

2. Construction of retroviral RNAi expression vector expressing a chimeric tetracycline repressor-Kruppel Associated Box (KRAB) supersuppressor. (completion of task two)

3. Introduction of hairpin sequences for genes targeted for regulated suppression into the vector and confirmation of the vector sequence. This was the goal of task three. This has been done for Prostate Derived Factor (PDF) and the tumor suppressor, p53.

4. The goal of task four was to assess the efficacy of the vectors for suppressing targeted gene expression in prostate cancer cells. This was done for p53 and for two different sequences targeting PDF.

5. Beyond the tasks proposed, additional iterations of the vector were constructed and tested in order to improve the level of gene suppression we were able to achieve.

Reportable outcomes: W81XWH-04-1-0205 Development of vectors for the regulated expression of RNAi

A. Development of an LNCaP prostate cancer cell line with partial suppression of PDF expression in response to tetracycline. See figure 2.

B. The following vectors were constructed.
1. pU6O/RK
2. pU6O/RK-PDF
3. pU6O/RG
4. pU6O/RG-PDF
5. pU6O/RG-PDF2
5. pU6O/RG-p53
6. pU6O*/RG

Conclusions: While the vectors proved to be facile to use experimentally, the degree of suppression of gene expression we were able to observe was somewhat disappointing. For LNCaP cells where PDF was targeted, some clones exhibited a fairly robust suppression in response to tetracycline but many did not. For p53 no satisfactory clones were identified even though we confirmed that we were getting good expression of the tet-repressor fusion protein. A final iteration of the vector was developed which has binding sites for the tet-repressor purported to be superior to the widely used consensus. This could yield stronger and more consistent suppression of gene regulation using this type of vector.

SUPPORTING DATA: W81XWH-04-1-0205 Development of vectors for the regulated expression of RNAi

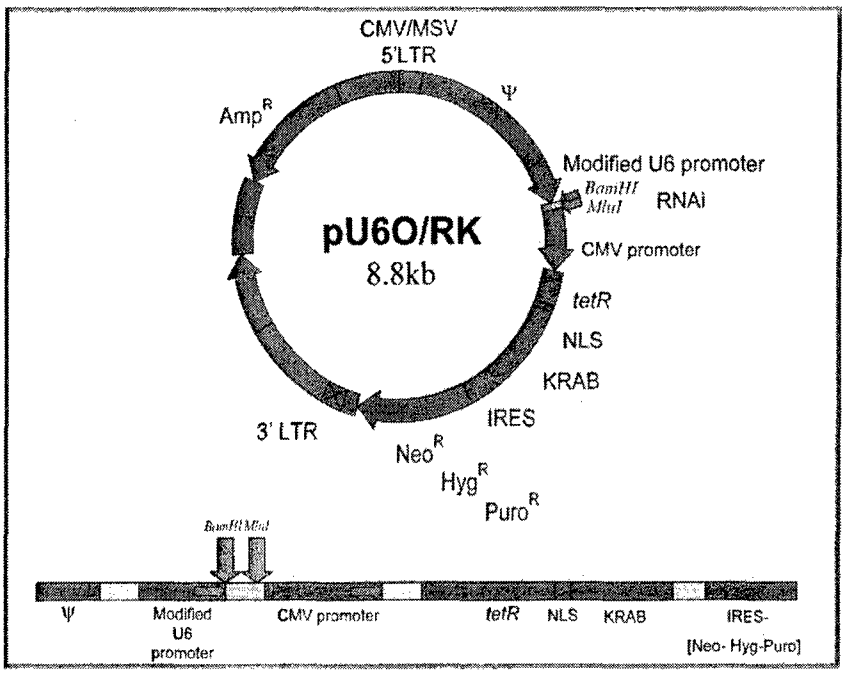

Figure 1. pU6O/RK, a vector for tetracycline-regulated production of hairpin RNAs for regulation of targeted gene expression. The DNA oligonucleotides encoding sequences for the desired hairpin RNAs are directionally cloned between unique BamHI and MluI sites downstream of the U6 promoter sequences. Note that the 3' long terminal repeat (LTR) sequences of the retroviral vector is inactivated so that the only functional polymerase II promoter in the integrated vector sequences following viral transduction is the CMV promoter driving the chimeric tet-repressor fusion gene.

Figure 2. PDF expression in a clone of LNCAP prostate cancer cells transduced with pU6O/RK-PDF is suppressed within two to three days of addition of tetracycline to the culture medium.